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INTRODUCTION

This laboratory has been interested in the biochemical mechanism of degradation of wool by fungi, particularly *Microsporum gypseum*. The present paper attempts to explain what happens when the fungus attacks the sulfur portion of the keratin molecule. While much has been accomplished in explaining the paths of sulfur metabolism in animals, as ably reviewed by Fromageot (1), no biochemical work on wool and sulfur metabolism of *M. gypseum* has been noted in the literature.

EXPERIMENTAL

A. Materials and Analytical Methods

Scoured sheep wool was defatted with alcohol and ether, rinsed in distilled water and redried at room temperature, It was then chopped by a single passage through a 40-mesh sieve in a Wiley Mill, which gave lengths of fiber averaging between 30 and 200 μ.

The fungus primarily used was *Microsporum gypseum* PQMD 196, which had been isolated from woolen fabric. Two other fungi were also used:—*Scopulariopsis brevicaulis*, Aust 69, and *Aspergillus niger*, PQMD 4j.

Four basic media were used throughout this work and are hereafter referred to by the numerals indicated below:

	I (Wool suspension)	II (Peptone-dextrose)	III (Proline-dextrose)	IV (Tyrosine-dextrose)
	g./l.	*g./l.*	*g./l.*	*g./l.*
$MgCl \cdot 6H_2O$	1.68	1.68	1.68	1.83
K_2HPO_4	2.09	2.09	2.09	2.21
KH_2PO_4	2.68	2.68	2.68	2.50
NH_4NO_3	—	—	—	3.00
Peptone	—	10.0	—	—
Dextrose	—	10.0	1.00	5.00
L-Proline	—	—	1.00	—
L-Tyrosine	—	—	—	5.00
Wool	50.0	—	—	—

Cystine and cysteine were determined by means of the Kassell and Brand (2) photometric method. Sulfur was determined gravimetrically as sulfate, and, in the case of solid residues, after oxidation in a bomb. Hydrogen sulfide was sought by absorption in cadmium acetate and examined quantitatively by means of the color produced by *p*-aminodimethylaniline monohydrochloride (diamine reagent). The method for determination of intermediate oxidation products was that of Lavine (3); thiosulfate by iodometric titration. The photometric method of McCarthy and Sullivan (4) was used for methionine assay.

B. Shake Flask Technic for Study of Action of Fungus on Wool

A 22-l. Pyrex round-bottom short ring-neck flask was used as the reaction chamber. A train for aeration was set up as follows: (a) air supply, compressed air; (b) large air filter capable of being sterilized in an autoclave; (c) cadmium acetate gas washer, to trap any hydrogen sulfide; (d) boric acid gas washer, to trap any ammonia vapors; (e) 10-l. Pyrex bottle containing 6 l. distilled water, to insure a moist air supply to the main reaction flask continuously throughout the experiment; (f) the main reaction flask; (g) two boric acid gas washers for the adsorption of ammonia. Cadmium acetate gas washers for the absorption of H_2S were omitted from the end of this train, since it had been shown repeatedly in preliminary experiments that no H_2S was given off as a result of the action of *M. gypseum* on wool or sulfur-containing amino acids.

The main reaction flask was filled with 5 l. of Medium I containing 5% of wool, which served as a sole source of C, N and S for the growth of the microorganisms. To insure sterile conditions before inoculation, that part of the train indicated by b, c, d, e, and f were sterilized for 1 hr. at 120°C. Prior to inoculation, the train was aerated for an hour to blow out gases arising from the partial decomposition of the wool during autoclaving (5).

Aeration was at the rate of approximately 600 ml./min. The reaction flask was mounted on a horizontal shaker which oscillated through a 4 in. path at the rate of 90 cycles/min. Samples were withdrawn aseptically while the appartus was moving after 4, 8, 11, 14, and 21 days growth, respectively.

The insoluble residue from the aliquot taken was separated by centrifugation, washed twice with 95% ethyl alcohol and then ethyl ether and dried to constant weight in a vacuum oven at 70°C. The mycelium was determined on 5 g. portions of the dried total solids. They were placed in a flask and brought just to boiling in 10% NaOH, whereupon all the wool and about 45% of the mycelium dissolved.

C. Metabolic Studies on Sources of Sulfur Other than That of Wool

Stock cultures of the fungi were grown in flasks containing nutrient solution II (peptone-dextrose). In the case of the experiments on cystine and cysteine and their partially oxidized derivatives, the quantity of mycelium used in each determination was the total of the 4 day growth at 30°C. of three 500 ml. Erlenmeyer flasks containing 150 ml. of nutrient solution each. To measure the end-products of metabolism on a known substrate, the fully-grown mycelium was washed aseptically by centrifuging, washing twice with sterile distilled water and finally resuspending in Medium III

(proline-dextrose). Although oxidation of cystine takes place with cells suspended in 0.5 *M* phosphate buffer, pH 7.0, a medium devoid of any source of N, C, and S other than the specific substrate added, autolysis of the cells seems to occur. This was indicated by the fact that the nitrogen in the filtrate was 2–3 times that of the nitrogen of the added substrate in the 0.5 *M* phosphate buffer. When substrate plus L-proline was used, however, the nitrogen in the filtrate equalled the sum of the substrate and proline nitrogen. When dextrose and proline were added as additional nutrients, an increase in weight of mycelium was noted, and when left out, a loss of weight resulted.

An aeration train, similar to that previously described, was used with a stationary 500 ml. round-bottomed reaction flask. A known quantity of substrate (cystine, cysteine, *etc.*) was placed in the reaction flask, the rubber stopper and glass tubing inserted, and the whole sterilized by autoclaving for 20 min. at 15 lbs. pressure. One hundred and fifty ml. of sterile nutrient solution III (proline-dextrose) containing the washed mycelium were added aseptically to the flask and aeration allowed to proceed. Results were discarded whenever contamination was observed.

Upon completion of a run, the mycelium was separated from the medium by centrifugation and washed with distilled water. The supernatant culture medium and washings were combined and made up to volume for subsequent analyses.

When working with methionine, it was found advantageous to increase the size of the equipment and to use a larger amount of cells, to aid in the greater production of the methyl mercaptan formed. The growth of cells from 1 l. of medium II (peptone-dextrose) was used in a 2-l. Erlenmeyer flask placed in the aeration train.

When the mycelium of the fungus was incubated with L-cystine, both 250 and 100 mg. L-cystine (1.04 and 0.42 millimoles) were used, and the results with either quantity were found to be almost identical in the recovery of metabolic products. Similar results were obtained when using 250 and 100 mg. L-cysteine (2.06 and 0.82 millimoles). In the case of L-cystine disulfoxide[1], 100 mg. or 0.37 millimole was used, and 100 mg. or 0.67 millimole of L-cysteine sulfinic acid[1] was used. The quantities of DL-methionine used are given in Table IV.

D. Effect of Source and Concentration of Sulfur on the Growth of M. gypseum

Medium IV (tyrosine-dextrose) was used, to which the sulfur sources and quantities indicated in Table III were added. Triplicate samples were placed on a shaker and allowed to grow for 13 days at 30°C. The mycelial growth was harvested by filtering through tared sintered glass crucibles of medium porosity. They were then washed and dried approximately 16 hr. at 100°C.

Results and Discussion

A. Sulfate Formation from Organic Sulfur of Wool by M. gypseum

Analyses of the wool used in these experiments, corrected for moisture and ash, showed 16.67% N, 2.69% S, and 8.00% cystine.

[1] These compounds were kindly supplied by Mr. Norman Floyd of the Lankenau Hospital Research Institute, Philadelphia.

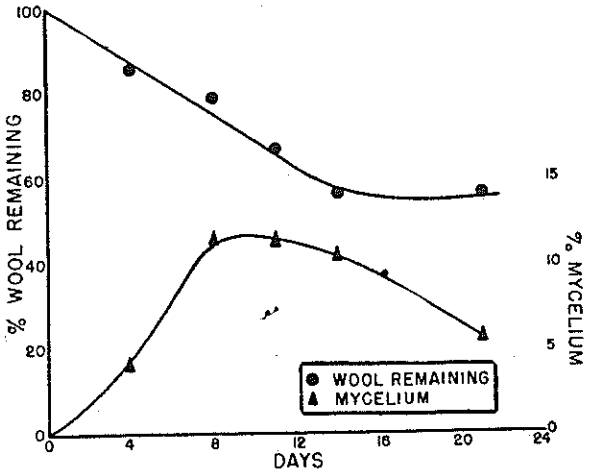

FIG. 1. Changes in wool and mycelium in shake-culture.

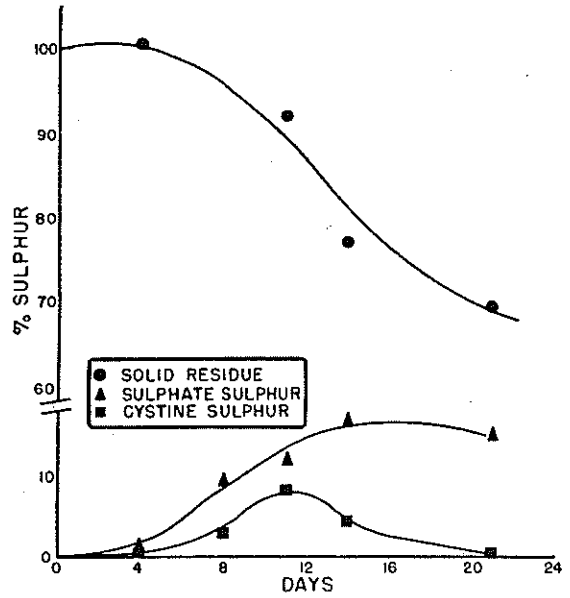

FIG. 2. Changes in the sulfur of wool degraded by *M. gypseum*.

TABLE I

Estimation of Metabolic Products Produced by M. gypseum on Various Organic Sulfur Compounds

Substrate	Time of aeration	Recovered as per cent. calc. in terms of substrate recovery						Total recovery
		Sulfate	Cystine	Cysteine	Cystine disul-foxide	Cysteine sulfinic acid	Hydrogen sulfide	
	hr.							
L-Cystine	4 c	0	96.4	0	0	0	0	96.4
	4 e	+	95.0	0	0	0	0	96.0
	18 c	0	96.0	0	—	—	—	96.0
	18 e	98.2	0	0	—	—	—	98.2
L-Cysteine	4 c	0	100.0	0	0	0	0	100.0
	4 e	6.4	82.5	0	0	0	0	88.9
	18 c	0	100.0	0	0	—	0	100.0
	18 e	32.6	59.2	0	+	—	0	91.8
Cystine disulfoxide	4 c	0	1.0	0	95.0	—	0	96.0
	4 e	15.7	32.2	0	41.5	—	0	89.4
	18 c	0	27.0	0	73.0	—	0	100.0
	18 e	77.8	2.5	0	0	—	0	80.3
Cysteine sulfinic acid	4 c	0	0	0	—	97.0	0	97.0
	4 e	3.3	0	0	—	89.8	0	93.1
	18 c	0	3.5	0	—	92.7	0	96.2
	18 e	66.0	0	0	—	0	0	66.0

— = not determined
0 = not detectable
+ = trace detectable
c = control
e = experimental

Analytical values for a known aliquot, corrected for evaporation losses, are plotted in Figs. 1 and 2. Maximum growth is found at 8 days, after which there is a gradual loss in dry weight of mycelium. The wool appears to be digested at a relatively steady rate until the 14th day, whereupon it levels off. Almost paralleling the loss of wool solids with time is the loss of S in the solids of the sample withdrawn, as indicated in Fig. 2. Actually there is an increase in the per cent of sulfur

of the residual wool, but it is not our intention to suggest what this might imply at this time. The sulfur calculated as cystine sulfur (obtained by direct quantitative cystine determination on the filtrate) rises to a maximum in 11 days and then gradually declines, almost entirely disappearing by the 21st day. Meanwhile, sulfate sulfur increases constantly, leveling off after the 15th day. The sulfate sulfur appears to stay constant at the expense of the cystine sulfur, indicating an oxidative enzyme system bringing about this conversion.

B. Metabolism of M. gypseum on Certain Forms of Sulfur Other Than That of Methionine

Averages of replicate experiments on the various substrates are summarized in Tables I and II. These data bring out the following

TABLE II

Estimation of Metabolic Products Produced by M. gypseum on Various Sources of Sulfur

Substrate	Time of aeration	Recovery as per cent, calc. in terms of substrate recovery					Total recovery
		Sulfate	Cystine	Cysteine	Thio-sulfate	Hydrogen sulfide	
	hr.						
	4 c	—	—	—	100.0	0	100.0
	4 e	0	0	0	98.2	0	98.2
Thiosulfate (+ 1% dextrose)	18 c	—	—	—	101.0	0	101.0
	18 e	0	0	0	99.8	0	99.8
Wool	7 days e	14.4*	3.5†	0	—	—	
L-Cystine (anaerobic conditions)	18 c	0	100.0	0	—	0	100.0
	18 e	0	100.0	0	—	0	100.0
Action of cell-free filtrate on cystine	18 e	0	98.0	0	—	0	98.0

— = not determined
0 = not detectable
+ — trace detectable
* = % of total sulfur in wool used
c = control
e = experimental
† = % of total cystine in wool used

facts:

1. The sulfur of cystine is only slightly converted by the fungus to sulfate at the end of 4 hr. but the oxidation is practically complete at the end of 18 hr.

2. Cysteine is completely oxidized to cystine in the absence of the fungus, simply by aeration. It is well known that traces of metals will catalyze the conversion. In the presence of the fungus a small amount of sulfate was formed, the remainder going to cystine at the end of 4 hr. However, at the end of 18 hr. approximately $\frac{1}{3}$ of the total cysteine has been oxidized to sulfate and the remainder to cystine.

3. Cystine disulfoxide, in the control, is slowly converted to cystine. However, in the presence of *M. gypseum*, sulfate appears. It has been established by Lavine (3) that aqueous solutions of cystine disulfoxide undergo dismutative decomposition, or hydrolytic oxidation and reduction, resulting in the formation of cystine and acid derivatives. The rate of decomposition increases with increasing pH. Thus, the sulfate that appeared was most likely the result of the oxidation of the cystine or the acid derivatives formed.

4. Cysteine sulfinic acid, as in the case of cystine, is only very slightly converted to sulfate in 4 hr., but all of the quantities accounted for converted in 18 hr.

5. Thiosulfate apparently is not further oxidized to sulfate under the conditions of the experiment. Armstrong (6) has shown that several fungi have this ability.

6. Under anaerobic conditions all the cystine was recovered as such, even after 18 hr.

The mycelium of *M. gypseum* possesses strong dehydrogenase activity as demonstrated by the Thunberg technic, using methylene blue. Cell-free filtrates from cultures grown on wool have no such activity, although they do have strong proteolytic acitvity. Cell-free filtrates cannot oxidize cystine in the presence of oxygen.

The data accumulated lead us to postulate tentatively the pathways of sulfur metabolism as diagramed in Fig. 3. Compounds appearing in brackets in Fig. 3 are postulated, since they have not been demonstrated in the metabolic filtrates. Cysteine is readily converted to cystine; whether this is autoxidation or enzymic catalysis cannot be stated from our data. Cystine is then hydrolyzed to cysteinesulfenic acid and cysteine. Sulfenic acid dismutes spontaneously into sulfinic

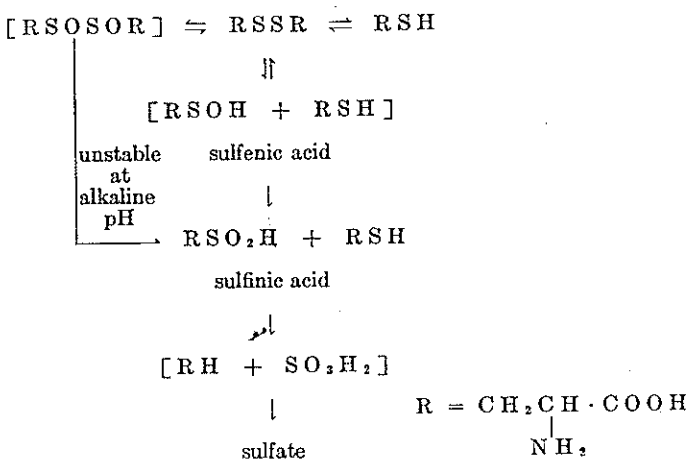

FIG. 3. Sulfur metabolism of *M. gypseum*.

acid and cysteine, according to the equation 2 RSOH→RSO_2H+RSH. Cysteinesulfinic acid rapidly gives rise to inorganic sulfate. When cystine disulfoxide is placed in the cycle, it too is rapidly converted to inorganic sulfate. The formation of cysteic acid and then taurine can be ruled out on the basis of practically 100% conversion of cystine to sulfate (7).

C. Inhibitory Action of Methionine on M. gypseum

Growth of the fungus in liquid culture containing different sources and concentrations of sulfur compounds are summarized in Table III.

TABLE III

Effect of Source and Concentration of S on Growth of M. gypseum (196)
mg. dry wt./culture

S source	Molar conc. of S				
	0.01	0.001	0.0001	0.00001	0
Cystine	63	70	14	13	—
Cysteine	55	67	16	11	—
Methionine	4	74	32	12	—
Na_2SO_4	65	65	41	11	—
Blank	—	—	—	—	12.5

Increasing quantities of cystine, cysteine, and sodium sulfate stimulated mycelial growth. After a certain concentration level no further changes occur, regardless of further increase of the sulfur compounds. However, at somewhere above 0.001 *M* methionine there is a strong inhibition of growth.

D. Fate of Methionine in the Metabolism of Fungi

While determining the effect of *M. gypseum* on several sulfur-containing amino acids, a highly objectionable odor similar to decaying cabbage was noted when methionine was used. In contrast to the use of cystine and cysteine, only traces of sulfate were produced. Part of this volatile substance produced when methionine was used, was isolated and identified as methyl mercaptan; still some odor persisted after absorption of the mercaptan through $Hg(CN)_2$. The results of the action of the various fungi in the presence of methionine as the only

TABLE IV

Estimation of Metabolic Products Formed by Several Fungi on Methionine

Microorganism	Conditions of the experiments					
	None	*M. gypseum*	None	*M. gypseum*[a]	*Scopulariopsis brevicaulis*	*Aspergillus niger*
Mg. substrate used	100	100	500	500	500	500
Millimoles used	0.67	0.67	3.36	3.36	3.36	3.36
Total liquid volume	150 ml.	150 ml.	1 l.	1 l.	1 l.	1 l.
Time of aeration	18 hr.	18 hr.	6 days	4 days	18 days	6 days
	Recovered as per cent, calc. in terms of substrate recovery					
Cystine	0	0	0	0	–	–
Cysteine	0	0	0	0	–	–
Hydrogen sulfide	0	0	0	0	tr	tr
Sulfate	tr	2	tr	tr	tr	0
Ammonia	0	+	–	+	+	+
Methyl mercaptan	0	+	0	+	+	+
Methionine	98	43	98	5	–	–

tr = trace

– = not determined

+ = present, but not determined quantitatively

[a] This experiment was repeated on 3 separate occasions with the same results.

sulfur source, as well as the quantitative and qualitative determination of products formed, appear in Table IV. The results with *Scopulariopsis brevicaulis* and *Aspergillus niger* agree, but *Aspergillus niger* data differ from that of Challenger (8), since this fungus brought about the formation of methyl mercaptan. It is to be noted that all experiments always showed a fission of the C—S linkage within 3 days, if at all. In the case of *M. gypseum*, 48 hr. after the start of formation of mercury methyl mercaptide crystals in the trap, no further production apparently ensued.

In those experiments where a total volume of 150 ml. of nutrient solution was used, the small amount of $Hg(SCH_3)_2$ formed appeared as crystals on the inside of the tube projecting into the $Hg(CN)_2$ gas absorber. The procedure was to carefully wash the crystals down into a small beaker, centrifuge off the supernatant and rewash with distilled water. They were then taken up in a very small amount of hot anhydrous alcohol and allowed to crystallize. They were then recrystallized 3 times in this manner. When using a total volume of 1 l. of nutrient solution and 500 mg. DL-methionine in the experiments, relatively large amounts were recovered. The crystals appeared as large thin shining plates, turning greenish-brown at 160°C. and melting with decomposition at 174°C.; they did not depress the melting point of an authentic specimen, m. pt. 175°C. Found: S, 21.40%, 21.47%; $Hg(SCH_3)_2$ requires 21.75%.

These findings were of interest for several reasons. It has been shown by Birkinshaw, Findlay and Webb (9) that the fungus *Schizophylum commune* had the ability to form methyl mercaptan (alkyl sulfide) from inorganic sulfate. The keratinolytic fungus, *M. gypseum*, with which we have been working, does not effect this synthesis when grown on inorganic sulfate or thiosulfate. Steinberg (10), working with *Aspergillus niger* and with inorganic sulfate and its products of reduction, has shown that soduim sulfoxylate (Na_2SO_2) is the lowest state of oxidation in which inorganic sulfur can be utilized efficiently, and that sulfide and disulfide were not utilized. Further, assimilability of organic sulfur varied with molecular configuration and was correlated with the presence of attached or adjacent oxygen in the molecule. Cystine, homocystine and methionine were readily available as sole sources of sulfur supply.

Challenger (8) has noted the ability of several fungi to cause fission of the —S—S— link of organic disulfides, R—S—S—R. giving the thiol R—SH and the alkyl methyl sulfide R—SMe. He also took many other organic sulfur sources and was able to show the C—SMe linkage fission. These last examples, involving methylation of organically linked sulfur, are in a somewhat different category from the direct methylation of

inorganic sulfur, as first shown by Birkinshaw *et al.* (9). This fission of the alkyl $-S-C$ link is a type of microbiological action which presents analogy with the fission of cystathionine in animal tissues.

Summary

1. Degradation of wool by the action of the fungus *Microsporum gypseum* was followed analytically with respect to sulfur, using the shake-culture technic.

2. With time, there was a decrease in the sulfur in the solid-residue aliquot with an increase in the sulfate sulfur, which levels off after 13 days and stays constant. The cystine sulfur in the filtrate rose to a maximum along with the sulfate sulfur until the 11th day, whereupon it decreased, so that it was practically absent at the 21st day.

3. Inorganic sulfate is an end-product of the decomposition of wool by fungi.

4. A scheme for the metabolism of cystine and cysteine has been suggested, wherein any cysteine is converted to cystine, which, in turn, is enzymically hydrolyzed to cysteinesulfenic acid, the latter being oxidized to cysteinesulfinic acid, and finally to the sulfate ion.

5. *M. gypseum* has the ability to cause fission of the $C-S$ linkage in methionine with the production of methyl mercaptan.

6. Methionine, as contrasted with cystine, cysteine, and sodium sulfate, caused a very definite inhibition of growth of the fungus at high concentrations. This was ascribed to the formation of toxic methyl mercaptan.

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